IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Akihiko YAMAGISHI
SERIAL NO: 09/897,107 Group Art Unit: 1652
Serial No. 09/839,214 Examiner: Ramirez, Delia M.
Filed: July 3, 2001

#15
D.G.J
7/9/03

For: METHOD FOR IMPROVING THERMOSTABILITY OF PROTEINS, PROTEINS HAVING THERMOSTABILITY IMPORVED BY THE METHOD AND NUCLEIC ACIDS ENCODING THE PROTEINS

DECLARATION UNDER 37 C.F.R. 1.132

Honorable Commissioner
of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Akihiko YAMAGISHI, inventor of the above-captioned application, do hereby declare as follows;

The applicability of the method of the present invention to various proteins were determined by the following methods.

Thermal stabilization of glycyl tRNA synthetase (GlyRS) of *Thermus thermophilus* by an ancestral type mutation

1. Isolation of genomic DNA of *Thermus thermophilus*

T. thermophilus (HB8) was cultured overnight at 70°C in a 50 ml NM medium. The collected bacterial cells were lysed in TE (100 mM Tris, 10 mM EDTA), followed by disruption of the bacterial cell membrane by an operation such as lysozyme treatment, SDS treatment, or freeze thawing using liquid

nitrogen. The resulting extract was subjected to the phenol/chloroform: isopropanol (24 : 1) extraction to separate only nucleic acid. Thereafter, RNaseA was added thereto to degrade the RNA. After the phenol/chloroform: isopropanol extraction, ethanol was added thereto, and thus precipitated genomic DNA was rolled up with a glass rod and dried. Finally, the genomic DNA was dissolved in a 2 ml of TE solution.

2. Cloning of GlyRS gene

The primers presented below were designed on the basis of a genomic sequence of *Thermus thermophilus*.

Primer 1 5'-AAAAAAGCTTCATATGCCAGCATCAAGATTGGACGA-3'

Primer 2 5'-AAAAGAATTCTACCACCTAAGCCTCTCCCG-3'

A mutation was induced in the primer 1 so that the third letter in the codon is changed into **A** (adenine) for the purpose of elevating the expression efficiency. Upon such a mutation, one of the amino acids was also modified, i.e., amino acid residue at position 5 changed from Ser to Arg. Using these primers and 2.5 ng of the genomic DNA of *T. thermophilus* as well as Ex *Taq* polymerase, the GlyRS gene was specifically amplified by PCR. The condition was: incubation at 94°C for 5 min, followed by 30 cycles of incubation at 94°C for 30 sec, 65°C for 30 sec and 72°C for 3 min, and thereafter at 72°C for 7 min. The amplified GlyRS gene was treated with restriction enzymes *Hind*III and *Eco*RI, and ligated into a vector pUC18 using

the ligation Kit Ver.2 (Takara Bio) (Figure 1). JM109 was transformed with thus resulting plasmid vector, and some of the grown colonies were selected, and extracted the plasmid thereof. An insert fragment of the plasmid was then confirmed. Among the clones confirmed to contain the insert, 4 clones were selected, and nucleotide sequence was determined by using ABI PRISM 377 DNA Sequencing System. In order to exclude the occurrence of error mutation upon PCR, a consensus sequence was produced from the 4 sequences, and thus clone No. 13, which was found to be 100% identical to the consensus sequence, was decided to be used in the following analyses and experiments. When the nucleotide sequence of the clone No. 13 and the nucleotide sequence in a database (AJ222643) were compared, differences were found at some parts (15 bp out of 1521 bp). The sequence of the clone No. 13 was converted to an amino acid sequence, and this amino acid sequence was compared with an amino acid sequence in a database (P56206). Accordingly, differences in 17 amino acids were found (Figure 2). On the basis of the comparison with amino acid sequences of α2 type G1yRSsfrom other organisms, it was presumed that the sequence on the database included errors. It was speculated that to analyze nucleotide sequences of thermophile such as *T. thermophilus* which had high GC content should be difficult in the time period of 1980s when the sequence of this database was determined.

3. Production of phylogenetic tree and estimation of ancestral type amino acid residues

The amino acid sequence of the clone No. 13 determined this time was aligned with amino acid sequences of α2 type GlyRSs from a variety of organisms obtained from DNA databank by ClustalX program. A model was worked out to assess how amino acid substitution occurred with a maximum likelihood method using a puzzle program, and thus a phylogenetic tree was produced using the Neighbor-Joining method with Phylip program. Figure 3 illustrates a phylogenetic tree produced for the α2 type GlyRSs. Because α2 type GlyRSs alone are used for this phylogenetic tree, it is an unrooted phylogenetic tree. Therefore, the position of a commonote can not be precisely determined. However, with reference to phylogenetic trees produced on the basis of 16S rRNA, the commonote is presumed as being positioned anywhere between the roots of Bacteria and Archaea/Eucarya. Thus, ancestral type amino acid residues were estimated using codeML in the Phylogenetic Analysis by Maximum Likelihood (PAML) programs based on information of the produced phylogenetic tree. Specifically, the sequences of the roots of Bacteria and Archaea/Eucarya, i.e., respective ancestor sequences were estimated. Next, the respective estimated amino acid residues were compared one by one. When these were the identical residues, it was assumed that "The

commonote also included the amino acid residue". Accordingly, estimation of an amino acid sequence assumed as being carried by the commonote was conducted (Figure 4). Referring to Figure 4 (1) as an illustrative example, in an estimated ancestral sequence of Archaea/Eucarya, the amino acid residue at position 30 is **A**, and the corresponding amino acid residue in the ancestral sequence of Bacteria is also **A**. Thus, it is suspected that the commonote also carried the amino acid residue **A** in this region. To the contrary, the amino acid residue of the corresponding region of *T. thermophilus* is **Q**. In other words, in respect of the amino acid residue at position 30, it can be considered that the common ancestor carried **A**, whilst it had been changed to the amino acid residue **Q** when divergence had occurred to raise the organism, *T. thermophilus*. Therefore, an attempt was made to approximate GlyRS of *T. thermophilus* to that which is assumed as being carried by the commonote, by changing the amino acid residue of this region from **Q** to **A**. In such a manner, 15 positions were selected as residues to examine the ancestral reversion. In addition, conservation of each of the amino acids was also approached. More specifically, amino acids positioned in a region with relatively high conservation in its vicinity were selected as target residues for the ancestral reversion. Such selection results from the fact that parts with lower conservation tend to have lower reliability for the estimation of the ancestral

sequence in their residues, which are inadequate as a target residue for the ancestral reversion. Moreover, because it is probable that adjacent residues concurrently developed, they were determined to subject to mutagenesis all together (Figure 4 (2), (3), (6) and (7)). As a result, 9 types of ancestral type mutated enzymes were planned to be produced as shown in Figure 4. Accordingly, primers for site-directed mutagenesis were designed. Sequences of the primers are as listed below.

Primer 3 (1) 5'-GTAGTCGTAGACGCCC**GC**AAGCCCCCCGTAGAT-3'

Primer 4 (2)

5'-GTCCTCCACGGGGCC**GA**T**G**TAGGTCTTGAACATCA**G**GTTGAAGTAGCGGGG-3'

Primer 5 (3)

5'-GAAGGGTAGCTTGCGGC**G**CGTGGC**T**TCCAGGACGTTCTTGAA-3'

Primer 6 (4) 5'-CTGCTCAAACTCCCG**GGT**CCGGAAGATGAAGTT-3'

Primer 7 (5) 5'-CCCCGGGCGGACGAAG**A**ACTCTATTTCCATCTG-3'

Primer 8 (6)

5'-GCCAACCGCACGGACT**A**TGACCTG**TCT**AGCCACACCAAGGAC-3'

Primer 9 (7)

5'-TACGAGGACACCGGC**TCC**ATCGGCAAG**CG**CTACCGCCGCCA**G**GACGAG**A**TCGGCA CGCCCTTC-3'

Primer 10 (7') 5'-GGCACGCCCTTC**TGC**GTCACCGTGGACTAC-3'

Primer 11 (8) 5'-AAGGACACGGTCACG**A**T**C**CGGGACCGGGACACC-3'

Mutation was induced at the base denoted with underline. (1) through (5) were designed as antisence primers, and (6) through

(8) were designed as sense primers. Upon the designing, codons for the mutated region were replaced with a reference to codon usage of *E. coli* (Andersson, Siv G. E. and Kurland, C. G. *Microbiol. Rev.* **54**, 198-210, 1990). Accordingly, amino acid substitutions: (1) Q30A, (2) M168L/V174I, (3) D203E/S206R, (4) V233T, (5) Y243F, (6) F315Y/G318S, (7) N440S/A444R/H448Q/V451I, (7') A456C, and (8) V480I are introduced.

4. Production of ancestral type mutant

Using a site-directed mutagenesis method by PCR (Picard, V., Ersdal-Badju, E., Lu, A. and Bock, S. C., **22**, 2587-2591, 1994), an ancestral type mutant was produced. The DNA used as a template was prepared by ligating the GlyRS gene (clone No. 13) that was inserted into pUC18 to an expression vector pET21c at an *Nde*I, *Eco*RI site to give a final concentration of 3.6 fmol/μl, as shown in Figure 5. PCR was carried out with the composition and under the conditions as shown in Figure 6. Consequently, amplification was found for all the mutants. These were treated with restriction enzymes *Nde*I and *Eco*RI, ligated to pET21c vector (Figure 7), and used for the transformation of JM109. Plasmid of thus grown colony was extracted, checked for the insert, and the entire nucleotide sequence was confirmed. As a result, although (2) and (6) included error mutations in the region other than the intended

mutation region, other ancestral type mutants were confirmed to have a correct nucleotide sequence in their entirety. PCR product of the ancestral type mutant gene which was confirmed as having a correct nucleotide sequence was ligated to an expression vector pET21c at an *Nde*I, *Eco*RI site as shown in Figure 7. JM109 was transformed with thus resulting product, and insert check was carried out for the grown colony. The nucleotide sequence was confirmed, and sequences of 7 ancestral type mutant genes (mu1, mu3, mu4, mu5, mu7, mu7', and mu8) were determined.

5. Expression and purification of ancestral type mutated enzyme

Bacterium for expression, BL21(DE3)pLys (Novagen), was transformed with wild type or each 7 ancestral type mutated enzyme plasmid. Thus grown colony was checked for insert, and thereafter, subjected to 2 L large-scale culture in 2xYT medium. When OD600 reached to 0.7, IPTG was added to give a final concentration of 1 mM. After 3 h from addition of IPTG, bacterial cells were collected. The collected bacterial cells were lysed in an extraction buffer. Composition of the extraction buffer is as follows: 250 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 5.0 mM Mercaptoethanol, 0.1 mM EDTA, 1 mM PMSF, 5 μg/mL Pepstatin, 5 μg/mL Leupeptin (Mazauric, M. -H., Keith, G., Logan, D., Kreutzer, R., Giege, R. and Kern, D., *Eur. J.*

Biochem. 251, 744-757, 1998). Thereafter, the bacterial suspension was sonicated to disrupt the cell membrane, and the bacterial homogenate was subjected to ultracentrifugation (4°C, 30000 rpm, 30 min). The supernatant was subjected to a heat treatment (70°C, 30 min) to denature heat unstable proteins. Then, the solution was again subjected to ultracentrifugation (4°C, 30000 rpm, 30 min) to remove the denatured proteins and the like which were precipitated. Thus resulting supernatant was applied to DEAE-Sephacel column (anion exchange column), Pharmacia Biotech (elution velocity: 4 mL/min, adsorption buffer: 20 mM PKi (pH 7.2), elution buffer: 20 mM KPi (pH 7.2), 0.4 M NaCl). When the supernatant from the ultracentrifugation was applied to the column, it was diluted by three-fold using the adsorption buffer as a solvent for the purpose of decreasing the salt concentration. Next, thus resultant fraction was confirmed on SDS-PAGE, and dialyzed in 20 mM KP (pH 7.2). Thereafter, the dialyzed liquid was applied on Resource Q column (anion exchange column), manufactured by Pharmacia Biotech (elution velocity: 4 mL/min, adsorption buffer: 20 mM KPi (pH 7.2), separation by a concentration gradient of 0 - 0.4 M NaCl). Finally, the separated liquid was applied on Resource 15PHE column (hydrophobic column), manufactured by Pharmacia Biotech to purify GlyRS (elution velocity: 4 mL/min, adsorption buffer: 20 mM KPi (pH 7.2), 20% ammonium sulfate separation by a concentration gradient of 20

- 0% ammonium sulfate). The results of the purification are illustrated in Figure 8. Figure 8 illustrates only the results of SDS-PAGE for one mutated enzyme, however, all of the wild type GlyRS and the ancestral type mutated enzymes which had been expressed and purified exhibited entirely similar results. From the results of SDS-PAGE on each of the steps in such purification, it was confirmed that wild type GlyRS and the ancestral type mutated enzymes resulted in apparent single band.

6. Measurement of thermostability

As one evaluation of heat resistance, thermostability was measured with CD. There was a possibility that the CD measurement could be affected due to a slight amount of residual glycerol even though dialysis was conducted, because large amount of glycerol was added to the protein preservation buffer to stabilize a protein and to avoid aggregation observed under a condition of 20 mM KPi (pH 7.2). Therefore, it was decided that all samples were measured after dialyzing with the condition as described below: 1) dilute the protein to 1.0 mg/mL with the preservation buffer; 2) dialyze in 1 L of 20 mM $NaHCO_3$ (pH 9.0) per 500 μL of the protein sample; 3) calculate each protein concentration with BCA Protein Assay Kit; and 4) adjust the concentration to 0.2 mg/mL. Thermostability of each sample at CD222nm was measured with Jasco J720

spectropolarimeter. Measurement at 222 nm was carried out for the purpose of identifying the denaturation in the protein in respect of the amount of the formation of α-helix. The temperature of the enzyme solution was controlled on a circulating bath equipped with a temperature controller (Neslab, Electron Co.), and the measurement was conducted with a themocouple, Keyence Corporation. The temperature was elevated from 50 to 130°C with an interval of 1°C/min. Average values from three measurement results are listed in Table 1. Among the 7 ancestral type mutated enzymes tested, two were unchanged in comparison with the wild type (mu5 and mu7'), and one exhibited decreased heat resistance (mu3). However, 4 of them could result in the elevation of heat resistance (mu1, mu4, mu7, and mu8).

Table 1: Thermostability of ancestral type mutated enzymes

Mutation No.	Tm
mu1	68.4
mu3	64.9
mu4	70.2
mu5	66.2
mu7	68.9
mu7'	66.2
mu8	67.0
wild	66.2

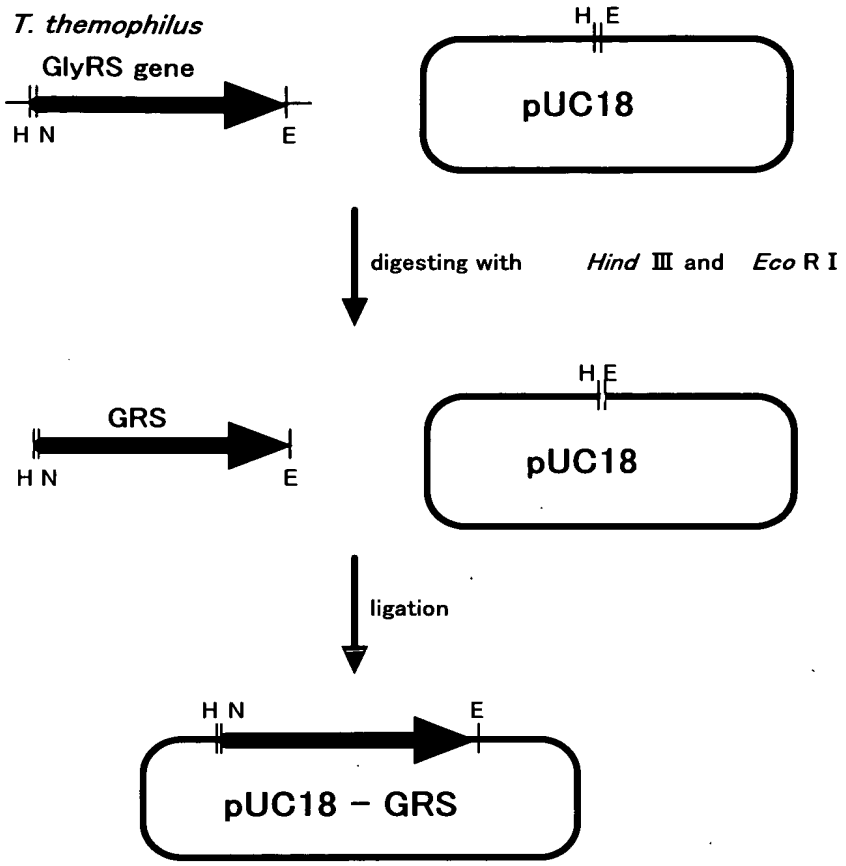


Figure 1. Cloning of GlyRS gene of *T. thermophilus*

GRS: *T. thermophilus* GlyRS gene (*grs*), H: *Hind*III site, E: *Eco*RI site, N: *Nde*I site

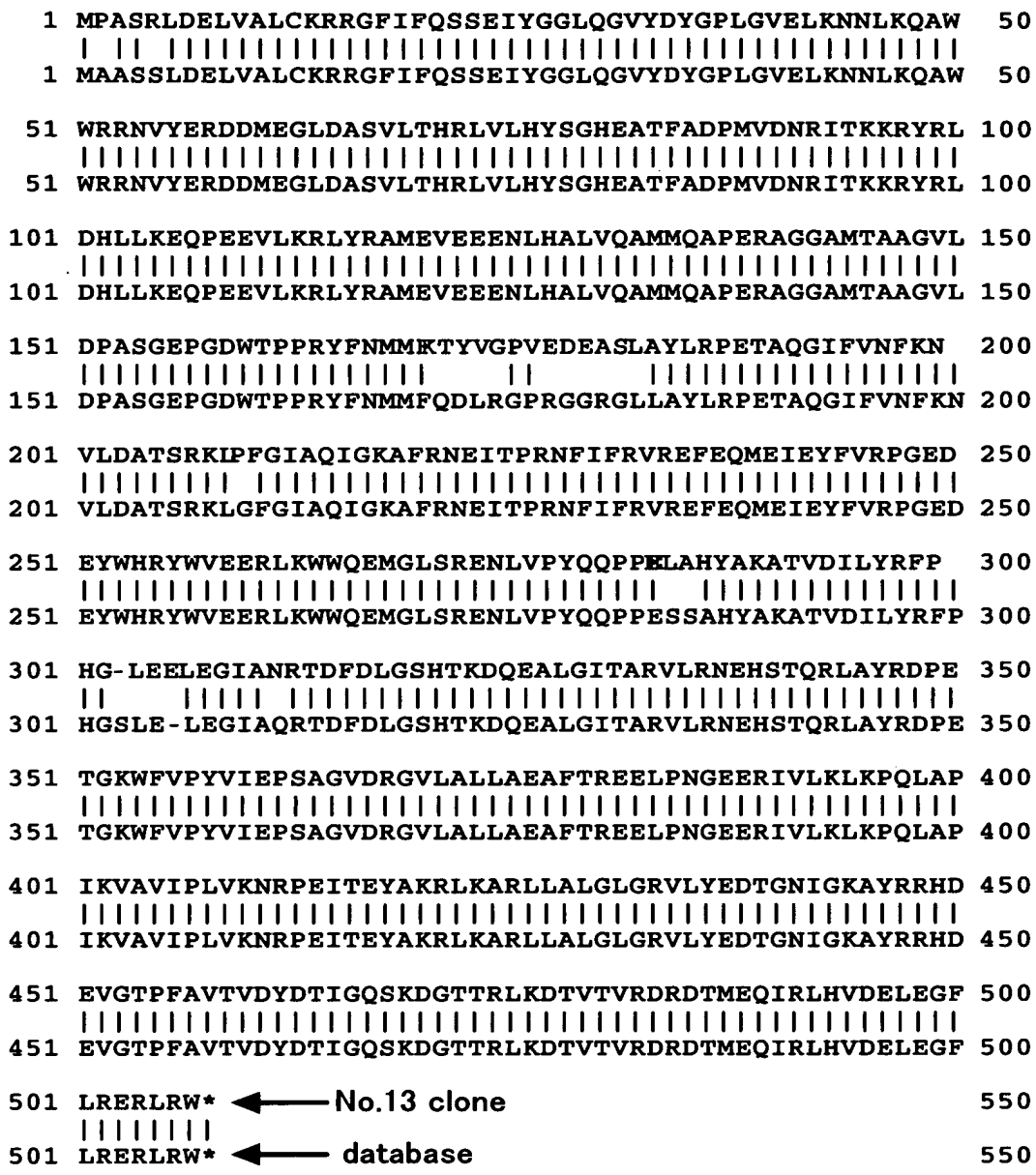


Figure 2. Determination of amino acid sequence of *T. thermophilus* GlyRS (type α2)

Blue colored sequence denotes the sequence of No. 13 clone; and black colored sequence denotes the registered sequence on

a database (P56206). Green colored residues denote the position with a difference found between No. 13 clone and P56206, and red vertical line denotes the position with the identical amino acid residues therebetween. The fifth residue (R) depicted by purple color is a position where modification of the amino acid residue was executed for elevating the expression efficiency.

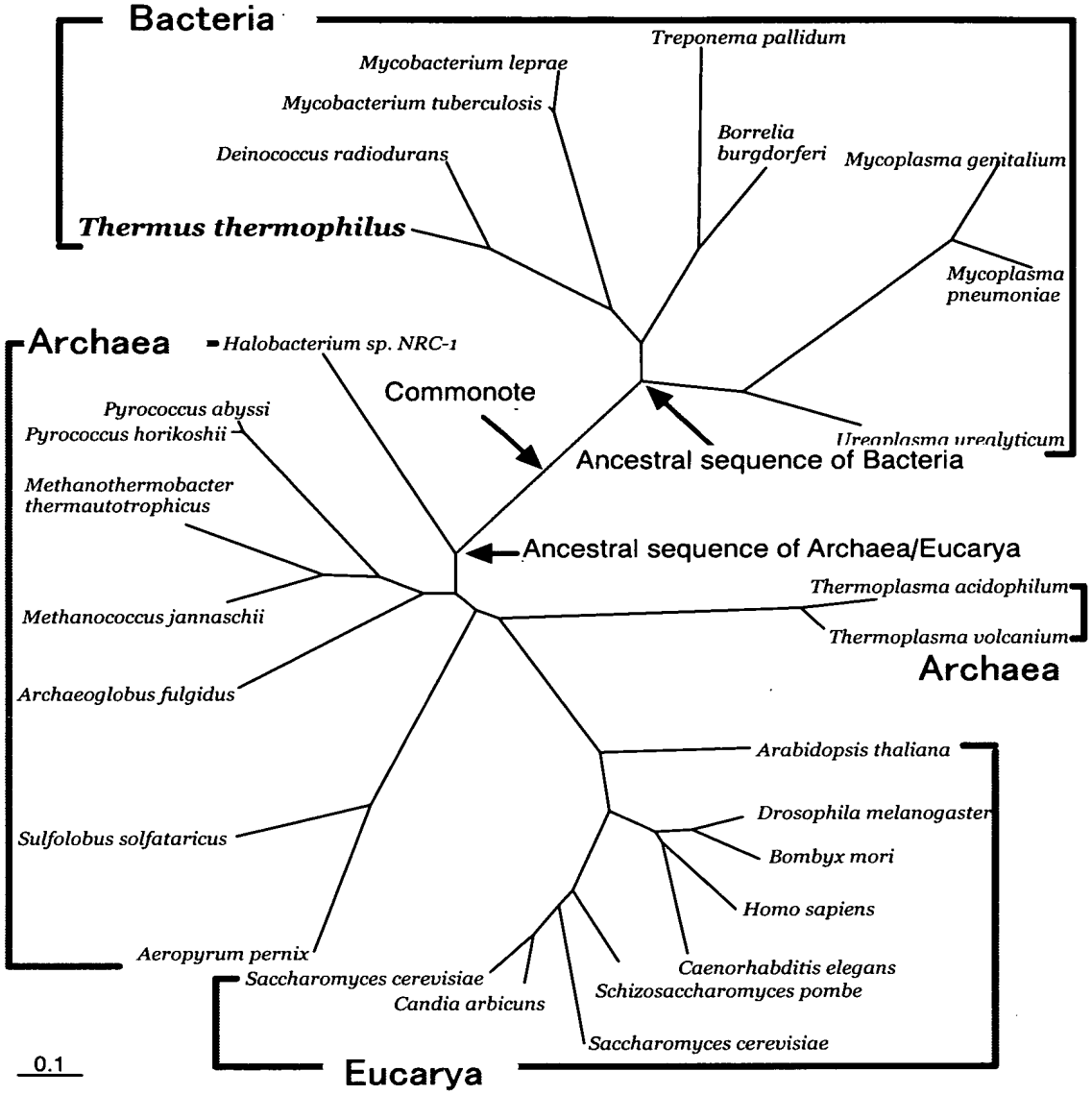
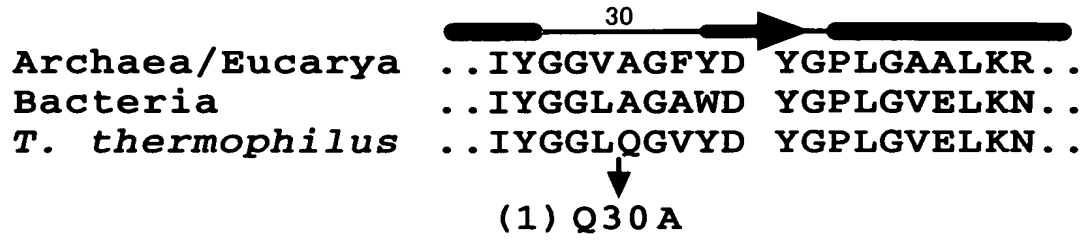


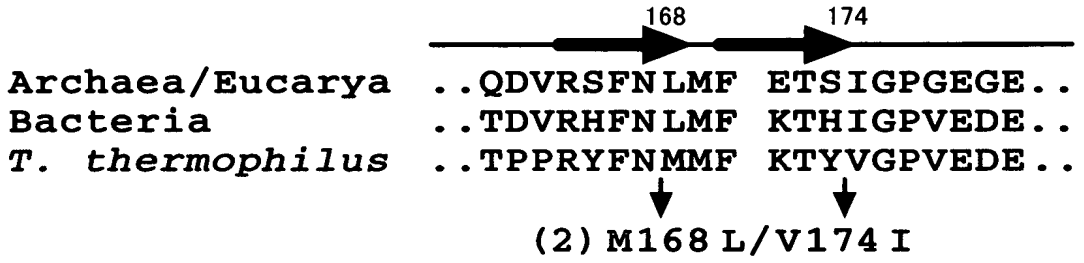
Figure 3. GlyRS phylogenetic tree of α2 type

Unrooted phylogenetic tree of α2 type GlyRSs is illustrated. The positions of the ancestral sequence of Bacteria and the ancestral sequence of Archaea/Eucarya are denoted by a purple arrow head. The position of the commonote is presumed to be located between the roots of Bacteria and Archaea/Eucarya, in other words, anywhere on the line depicted by green color.

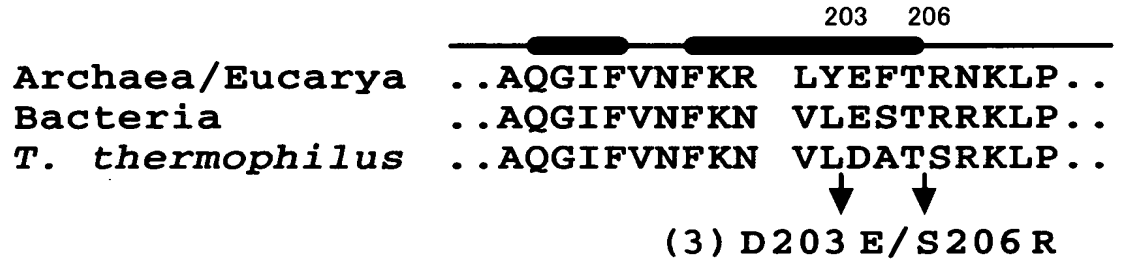
A



B



C



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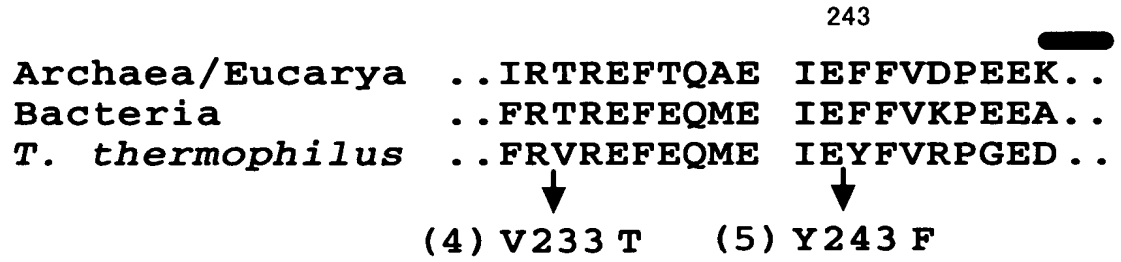
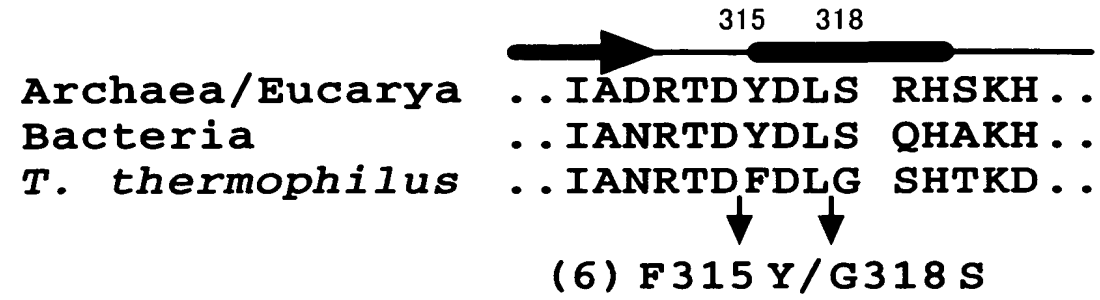


Figure 4. Continued

E



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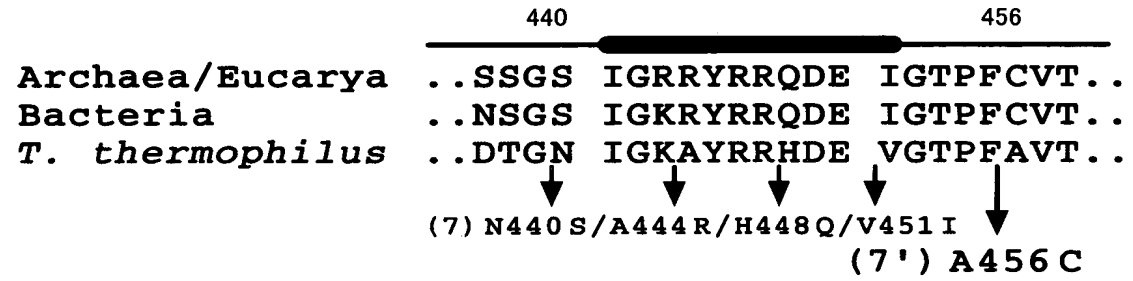

G

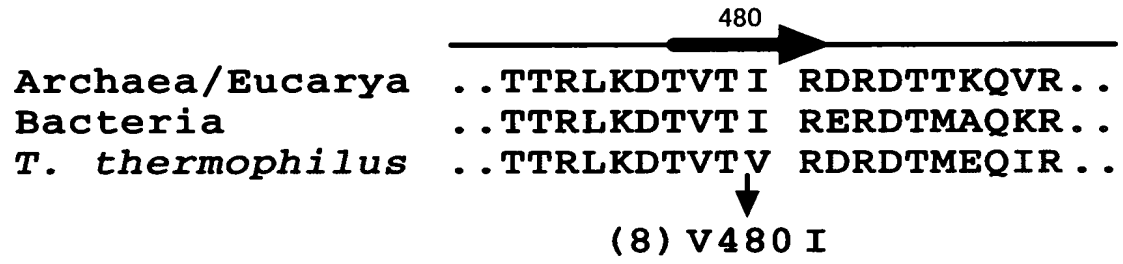

Figure 4. Estimation of ancestral type amino acid residues

Secondary structure is shown above the respective sequences. α-helices are denoted by a black box; 310-helices are denoted by a gray box; and β-sheet is denoted by a black arrow head. The numeral presented above the sequence is an amino acid residue number of *Thermus thermophilus*.

Archaea/Eucarya and **Bacteria** denote the ancestral sequence respectively estimated. Amino acid substitutions shown below the sequence were conducted.

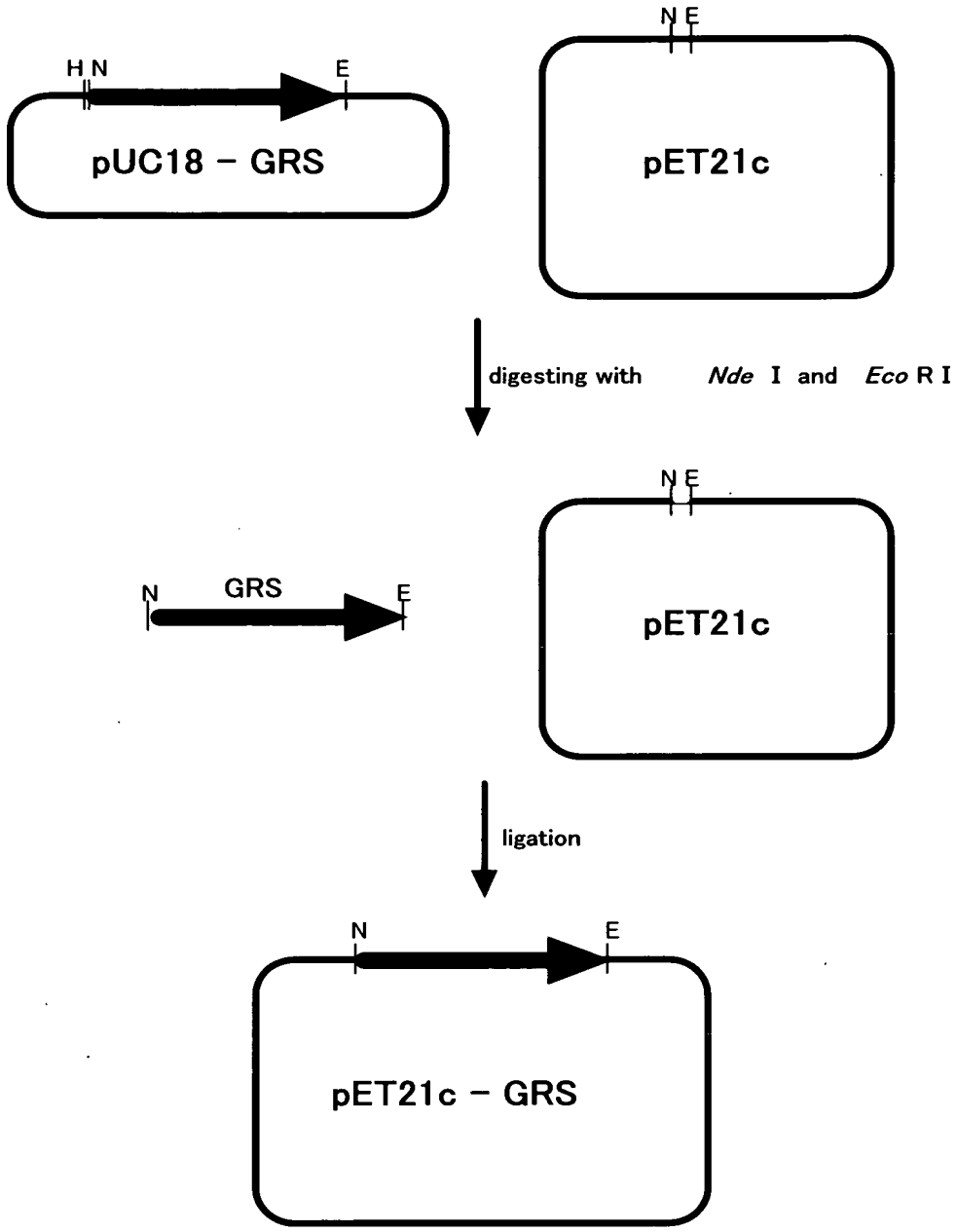

Figure 5. Transfer of GlyRS gene to expression vector pET21c

GRS: *T. thermophilus* GlyRS gene, H: *Hind*III site, N: *Nde*I site, E: *Eco*RI site

(Components)

template (3.6fmol/μL)	1μL
T7 P/T primer (10pmol/μL)	1μL
mutant primer (10pmol/μL)	1μL
dNTPmix (2.5mM)	8μL
2×GC buffer	25μL
D.W.	13.5μL
LA Taq polymerase	0.5μL
Total	50μL

(Conditions)

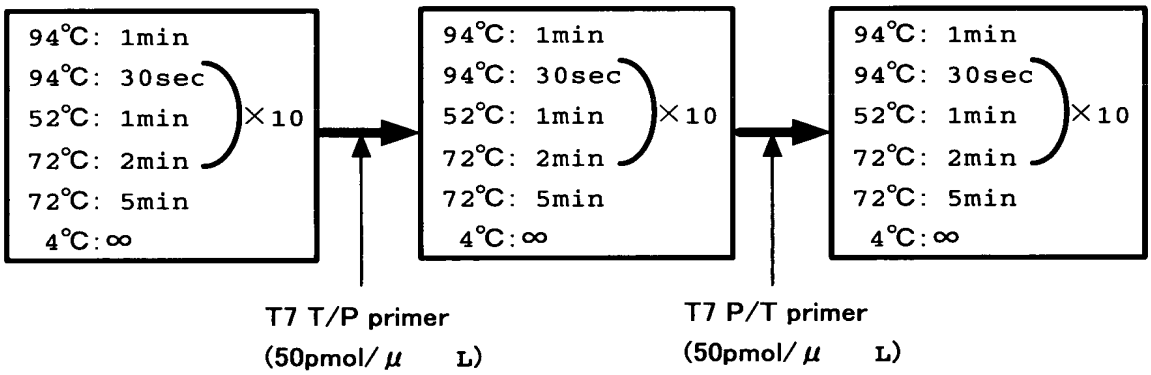

Figure 6. PCR condition for producing ancestral type mutant by site-directed point mutagenesis method

T7 P Primer: T7 promoter primer, T7 T primer: T7 terminator primer

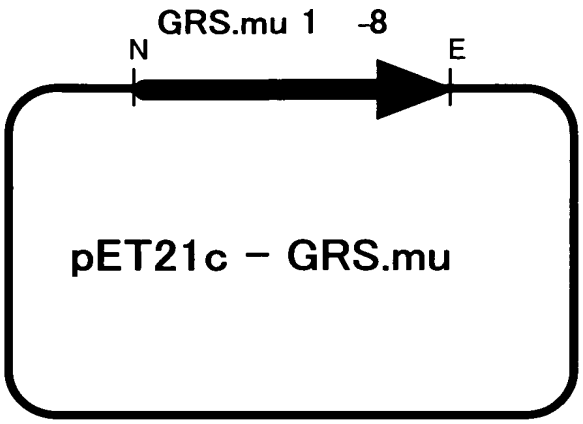

Figure 7. Production of ancestral type mutant plasmid

GRS.mu: GlyRS ancestral type mutant plasmid, N: *Nde*I site, E: *Eco*RI site

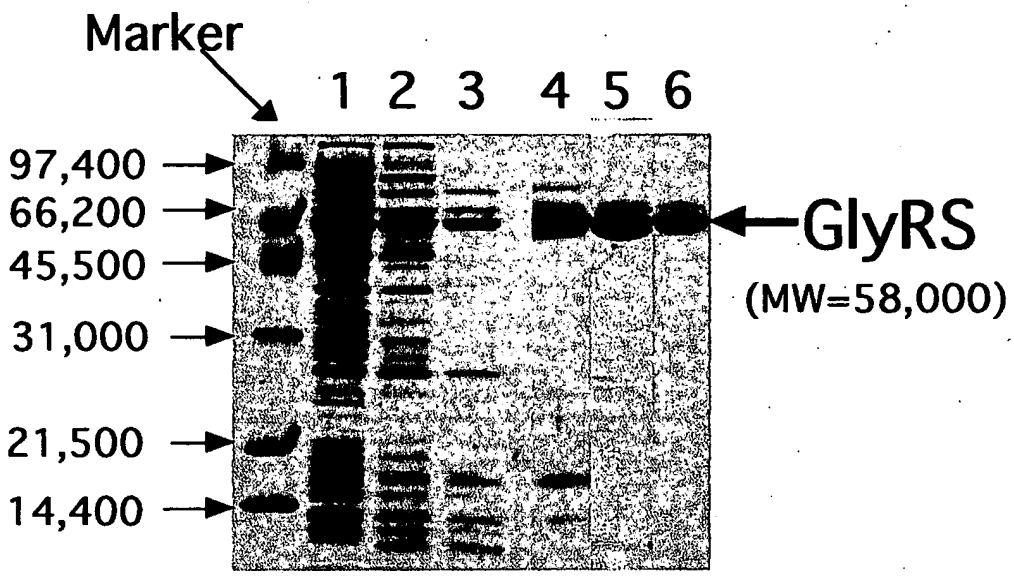

Figure 8. Purification of ancestral type mutated enzyme and wild type GlyRS

Numerals represent the purified samples in each of the following steps. 1. after sonication, 2. supernatant after the ultracentrifugation, 3. after heat treatment, 4. after DEAE-Sephace1, 5. after ResourceQ, 6. after ResourcePHE.

FURTHER, DECLARANT SAYETH NO.

Date: May 9, 2003 Name: _____

Akihiko YAMAGISHI